Exhibit 20

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DEVELOPING EFFICIENT STRATEGIES FOR THE GENERATION OF TRANSGENIC CATTLE WHICH PRODUCE BIOPHARMACEUTICALS IN MILK

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ABSTRACT

At the close of the millennium, a revolution in the treatment of disease is taking shape due to the emergence of new therapies based on human recombinant proteins. The ever-growing demand for such pharmaceutical proteins is an important driving force for the development of safe and large-scale production platforms. Since the efficacy of a human protein is generally dependent on both its amino acid composition as well as various post-translational modifications, many recombinant human proteins can only be obtained in a biologically active conformation when produced in mammalian cells. Hence, mammalian cell culture systems are often used for expression. However, this approach is generally known for limited production capacity and high costs. In contrast, the production of (human) recombinant proteins in milk of transgenic farm animals, particularly cattle, presents a safe alternative without the constraint of limited protein output. Moreover, compared to cell culture, production in milk is very cost-effective. Although transgenic farm animal technology was still in its infancy a decade ago, today it is on the verge of fulfilling its potential of providing therapeutic proteins that can not be produced otherwise in sufficient quantities or at affordable cost. Since 1989, we have been at the forefront of this development, as illustrated by the birth of Herman, the first transgenic bull. In this communication, we will present an overview of approaches we have taken over the years to generate transgenic founder animals and production herds. Our initial strategies were based on microinjection; at the time the only viable option to generate transgenic cattle. Recently, we have adopted a more powerful approach founded on the application of nuclear transfer. As we will illustrate, this strategy presents a breakthrough in the overall efficiency of generating transgenic animals, product consistency, and time of product development.

Key words: transgenics, milk, recombinant protein, cattle.

INTRODUCTION

Transgenesis can be defined as the alteration of genomic information with the intent to modify a specific physical trait of an animal. First applied in the early 1980s in mice, when genomic insertion (and subsequent expression) of the gene encoding human growth hormone resulted in enhanced growth (16), the addition or disruption of a host of different genes has yielded a flood of invaluable information regarding biomedical and developmental issues. Once transgenic mice could be generated with relative ease via pronuclear microinjection of recombinant DNA (9), attempts to modify the genome of other animal species soon followed (10). One of the most intriguing applications of transgenesis is the genetic modification of farm animals for production of (human) recombinant proteins in milk. The production potential of a livestock platform is formidable. Whereas 'small' farm animals such as pigs, goats and sheep

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produce between 300-900 L of milk annually, the yield of a single dairy cow can amount to ~10,000 L. Since the expression levels of recombinant protein in milk of transgenic animals routinely exceed 1 g/L (21, 25, 29), for every protein of therapeutic value, a limited number of animals would be required to meet patient needs. Particularly for (human) proteins that are scarce, production via transgenic animals would constitute a breakthrough. For example, the current supply of human blood clotting factors is still largely dependent on purification from donor blood, a source limited in availability and under constant threat of viral contamination. Due to the latter, frequent product recalls are not unusual. Although production of recombinant clotting factors in mammalian cell culture systems provides a safer alternative, the production capacity of this approach is limited. Moreover, mammalian cell culture systems are generally expensive (8), which is illustrated by the fact that therapy for hemophilia can amount to a hundred thousand dollars per patient annually. In comparison, transgenic cattle can not only safely produce therapeutics in milk at almost unlimited quantities, the economics of production (4) demonstrates it can do so at significantly lower costs.

TARGETED EXPRESSION IN THE MAMMARY GLAND

In order to generate transgenic cattle suitable for commercial production of therapeutic proteins, high-level expression, and tissue-specificity are of primary concern. Hence, the transgene should be combined with regulatory elements that drive efficient expression in mammary epithelial cells, specifically throughout lactation. The control elements most frequently used are derived from genes encoding abundantly expressed milk proteins, including ovine β-lactoglobulin (27), caprine β-casein (18), and murine whey acidic protein (WAP; 19). We have been using the regulatory sequences of the bovine αS1 casein gene. Over the years we have performed extensive studies in mice, rabbits, and cattle to verify the spatial and temporal expression pattern of transgenes driven by this promoter. Mammary gland specific transcription is detected immediately following the onset of lactation, whereas the transgenes are nearly silent in all other tissues examined[a]. In addition to the promoter sequences, the expression levels of recombinant proteins are also critically dependent on the composition of the transgene itself. The original genomic layout of a (trans)gene, including all it's exons and introns, appears far more effective than the corresponding cDNA sequence (17, 27). For example, Herman, the world's first transgenic bull carried a transgene cassette based on the αS1 casein promoter and the cDNA sequence for human lactoferrin (14). The concentration of this protein in the milk of Herman's transgenic female offspring never exceeded 0.01 g/L[b]. In contrast, transgenic cattle harboring a human lactoferrin transgene based on the protein's genomic composition have reached expression levels ranging from 0.4 up to 3.5 g/L[b].

GENOMIC INSERTION OF TRANSGENES VIA MICROINJECTION

Until recently, microinjection of recombinant DNA into the pronucleus of a fertilized oocyte remained the method of choice to generate a transgenic animal. First developed in mice (9), this technology was soon applied to other species, including rabbits, pigs, sheep (10), and

[a] Platenburg G; personal communication, manuscript in preparation.
[b] Van Berkel P, unpublished results.

cattle (14). The basic procedure for generating a transgenic animal is similar for most species. After oocyte maturation and fertilization, (linearized) DNA constructs encoding the recombinant protein of choice are injected directly into the pronucleus. Subsequently, the injected embryos are transferred to foster mothers. Combined data from a number of different laboratories show that the overall efficiency of microinjection as a technique to introduce recombinant DNA into the genome varies between species. Typically, transgenesis rates range from 15-30% for mice and 5-15% for pigs, rabbits and rats, to less than 5% for goats, sheep, and cows (26). Generation of transgenic animals is a very labor intensive and costly matter requiring large numbers of animals and dedicated facilities. This is particularly so for cattle, where only 1 or 2 injected embryos can be transferred to a single recipient heifer. Nevertheless, given their potential to produce large quantities of recombinant protein, we have performed several of such large-scale embryo transfer (ET) trials to generate transgenic (F0) cattle. In Table 1, the efficiencies of several of such trials are summarized. For example, in our first trial (ET-1), 21 pregnancies were established, resulting in the birth of 19 calves. Genetic analysis of the newborns demonstrated that two calves (the firstborn named Herman) had integrated multiple copies of the injected transgene DNA into their genome. Subsequent trials appeared to be less efficient, falling as low as 2.6 % (ET-3). However, none of these trials included a sufficient number of established pregnancies to be statistically significant. In all our trials combined, the transgenesis rate based on 342 pregnancies is 4.7%, which is nearly identical to the rate reported in other studies (7, 26). To increase the transgenesis rate, several studies were performed. One study involved collection of high-quality oocytes via ovum pick-up (OPU) for microinjection. A large number of embryos were generated after microinjection, and a limited number of these were transferred into foster mothers (ET-5; see Table 1). However, the transgenesis rate of non-transferred embryos (N.B. as determined by PCR analysis of extended embryonic cultures) and pregnancies combined was no higher than those of previous trials performed with 'slaughterhouse-derived' oocytes.

Table 1. Generation of transgenic cattle via microinjection.

Embryo transfer trials	No. Established pregnancies	No. Transgenic (%)
ET-1[a]	21	2 (9.5)
ET-3[a]	38	1 (2.6)
ET-5[b]	20	1 (4.0)
Total[a]	342	16 (4.7)
Ref: 7, 26	327	14 (4.3)

[a] Microinjection using slaughterhouse derived oocytes. Total includes ET-1, 3.
[b] Microinjection using oocytes obtained via ovum pick-up (OPU).

In addition to studies aimed at improvement of transgenesis rates, alternative ways were explored to use the available resources more effectively. For example, improved cattle management resulted in increased pregnancy rates. In addition, to minimize the number of non-transgenic pregnancies developing to term (generally >90%), a strategy for 'early transgene detection' was applied. For this purpose, transvaginal amniocentesis (TVAC) was performed to retrieve a small amount of amniotic fluid between Days 78 and 85 of gestation. This provides sufficient fetal material to verify, via the polymerase chain reaction (PCR), whether transgenes have integrated into the genome. As a consequence of the early termination of non-transgenic

pregnancies, the number of recipient heifers available for new transfers were maximized, and all calves born were transgenic.

SELECTION OF TRANSGENIC FOUNDERS FOR HERD ESTABLISHMENT

Not every transgenic founder animal is suitable for the establishment of a production herd. If promoter or protein encoding sequences of the transgene have been compromised during the integration process, the transgene will be essentially inactive. Intact transgenes can also remain silent if the genomic integration site is located in a heterochromatin region, which is transcriptionally inactive. Alternatively, integration close to highly active genes may have a boosting effect on the transgene expression level. Due to the unpredictable nature of such chromosomal position effects, determination of the recombinant protein concentration in milk is the only reliable method for selection of suitable founder animals. Generally, calving-induced lactation will not occur until the animal is ~2 years old. To speed up the selection process, a method for hormonal induction of lactation (15) can be used when the animals are between 2-6 months of age. Relatively small quantities of milk can be obtained for assessment of protein concentration, although we have also collected over 50 L from a single heifer by this approach[a]. In the latter case, sufficient quantities of recombinant protein can be purified for more extensive (functional) characterizations and pre-clinical tests.

EFFECTIVE GENERATION OF PRODUCTION HERDS

Since transgenic founder animals are hemizygous, no more than 25% of their offspring (i.e., the transgenic females), will be suitable to join a production herd. In practice, due to a phenomenon called mosaicism, the transgene transmission rate from founder to offspring often is lower than 25%. Mosaicism occurs if genomic integration of the injected transgene occurs after the first mitotic division, causing only a subpopulation of the cells to be transgenic. The distribution pattern of the transgenic cells in a mosaic animal is unpredictable; the degree of mosaicism can vary between tissues of the same animal.

If the germline is affected, the generation of a production herd from a mosaic founder bull, is a very inefficient process, particularly if conventional methods such as artificial insemination are used. To limit the number of recipient heifers required to breed a transgenic production herd, only female transgenic pregnancies should be established. Therefore, we apply a procedure to screen embryos prior to transfer. Briefly, high-quality donor heifers are superovulated and subsequently inseminated with transgenic sperm of a desired founder bull. Embryos are flushed from the uteri and graded based on their development stage. A small biopsy from the outer cell mass is collected from the embryos that qualify for transfer into recipient heifers. While the embryos are individually cultured in microdrops, a multiplex PCR analysis is performed on biopsies to identify the males (Y-chromosome) and the transgenics. Thus, all female transgenic embryos can be selected for transfer into synchronized recipient heifers. In Table 2, results of a typical herd generation program are shown for the generation of ~30 production animals (11). The transgene transmission rate of the bull in question had been determined prior to this campaign

[a] Salaheddine M, unpublished results.

at ~15-20% overall. Thus, breeding by conventional methods would have required the establishment of ~300 pregnancies. In comparison, after the pre-screening procedure, only 36 pregnancies had to be established to generate 29 female offspring; all of them transgenic.

Table 2. Pre-selection of transgenic production animals via PCR analysis of embryonic biopsies.

No. Transferable embryos/donors flushed	No. Embryos analyzed	Sex ratio (M/F)	No. Transgenic (M/F)	No. Pregnancies per embryos transferred (%)	No. Production animals born
602/112	562 (93%)	280/282	122 (22%) (67/55)	36/52 (69%)	29

GENERATION OF TRANSGENIC CATTLE VIA NUCLEAR TRANSFER

The relative inefficiency of microinjection as a tool to generate transgenic (farm) animals has sparked the development of many different approaches to distinguish transgenic from non-transgenic individuals. Such attempts include the coinjection of selectable marker genes (7) or pre-selection of transgenic embryos via PCR (3, 12). In addition, increased transgenesis rates have been achieved by alternative transgene delivery systems, the most promising being based on the use of retroviral vectors (1, 6, 13). Many of these methods are still under development and their success has been limited. Whether some of them will find their way to commercial application remains to be seen, as transgenic farm animal technology is being revolutionized by what promises to be a very powerful strategy: genetic manipulation of totipotent cells and generation of transgenic animals via nuclear transfer.

Nuclear transfer (NT) involves the introduction of the nucleus from a totipotent donor cell into a matured enucleated oocyte. The resulting embryo is transferred to a surrogate mother for development into a live calf. Key to the overall success of NT is the requirement for totipotency; defined here as the potential to initiate and direct normal development. Although many of the factors controlling totipotency have yet to be elucidated, blastomeres and various cell types from fetal and adult origin have been successfully used to clone a number of different species, including farm animals such as sheep (5, 28), goats (2, 24), and cattle (20, 22).

We derive totipotent cells from the genital ridge of female bovine fetuses that range in age between 40 to 60 days. These 'embryonic germ' (EG) cells are selectively cultured after enzymatic disaggregation of the genital ridge into individual cells (23). The recipient oocytes are routinely collected from slaughterhouse ovaries. After maturation, oocytes are enucleated by aspiration of the polar body and the metaphase plate. The actual transfer of the donor nucleus is achieved by electrical fusion of the totipotent EG cell with the enucleated oocyte. The feasibility of this procedure to generate cattle via nuclear transfer was demonstrated by the birth of the first cloned bull calf named Gene. When this technology is applied to generate several genetically identical cows, cloning can be valuable as a means of herd establishment. For the purpose of generating transgenic cattle, the critical breakthrough for an NT-based strategy lies in the pre-selection and expansion of characterized transgenic cells, prior to the generation of transferable

embryos. In addition, since EG cells used for genetic manipulation can be derived solely from female fetuses, only transgenic dairy cows with desired genetic merit for production are born.

The introduction of foreign genes (transgenes) into EG cells is achieved by standard transfection procedures. In order to facilitate the selection of cells that have correctly integrated the desired transgene(s) into the genome, a selectable marker encoding antibiotic resistance is typically included. Selection of transgenic cells based on antibiotic resistance is extremely effective. PCR results performed on selected cells typically yield greater than 90% positive for products requiring the integration of a single desired transgene[a]. For complex proteins that require the simultaneous integration of 2 or 3 different transgenes, we have achieved stable integration of all desired genes in >60% of the cells[a]. In addition to PCR, Southern blot analysis and fluorescent in situ hybridization (FISH) are used to verify integrity of the transgene structure and to determine copy number and integration site(s) of the transgene in the genome. This allows for selection and clonal expansion of cells carrying the desired number(s) of intact transgenes at favorable chromosomal locations, thereby ensuring the generation of cattle that express the recombinant protein of choice

As shown in Table 3, EG cells can be used quite successfully in NT for the production of non-transgenic cattle. However, for generation of transgenic cattle, any cell used for NT must undergo genetic manipulation and selection procedures combined with prolonged culturing in vitro, while remaining totipotent. In general, the number of times the cell has been passed and the number of days it has been maintained in culture are two of the many factors that can affect totipotency. By minimizing the impact of these factors the success of an embryo transfer program can be increased. In most cases, NT embryos derived from either transfected or non-transfected EG cells developed to transferable blastocysts in similar proportions (20%). Transfer of the transgenic embryos to recipient heifers has produced the first successes in our labs. Table 4 summarizes embryo transfer data from one of the transgenic cell lines that had undergone NT soon after transfection/selection procedures. For 50% of all transfers, pregnancy initiation was confirmed by ultrasound detection of a fetal heartbeat at ~28 days. The majority of the abortions occurred before the end of the first trimester. Following successful birth, transgenesis was confirmed for all 11 calves reported. The rates of pregnancy initiation and 'development to term' of this set of transfers are similar to results obtained with non-transgenic EG cells (see Table 3). However, the percentage of transgenic animals born (100%) is in sharp contrast to a microinjection-based approach (Table 1) which yields on average no more than 5% transgenic births.

Table 3. Generation of cattle via nuclear transfer.

No. Embryo transfers	Pregnancy initiation rate	No. Pregnancies to term	% Embryo transfers to term	% Initiated pregnancies to term
73	39 (53%)	14	19	35

[a] Forsberg E, Eilertsen K; personal communication.

Table 4. Generation of transgenic cattle via nuclear transfer.

No. Embryo transfers	Pregnany initiation rate	No. Pregnancies to term	% Embryo transfers to term	% Initiated pregnancies to term	No. Transgenic pregnancies
44	22 (50.0%)	11	25.0	50.0	11 (100%)

REDUCED TIME OF PRODUCT DEVELOPMENT

As demonstrated in the previous section, transgenic cattle can be generated far more efficiently via a strategy based on nuclear transfer. Due to the use of female totipotent cells for genetic manipulation and the subsequent selection of transgenic cells before nuclear transfer, all calves born will be female, transgenic, and non-mosaic. These advantages alone already constitute a major breakthrough compared to microinjection. An additional reason to apply nuclear transfer lies in the fact that a time consuming breeding process is not required to establish a production herd. Instead, herds consisting of genetically identical transgenic cows can be established instantly. For the development of therapeutic proteins, this is of significant importance, since it increases product consistency and cuts the "time-to-market."

To illustrate this, the timelines of different scenarios are outlined (Figure 1) for the production of ~100 kg of recombinant protein in milk of transgenic cattle. Assuming a single cow produces a total of 10,000 L of milk in a lactation period of ~8 months, and the concentration of the recombinant is 2 g/L, only 5 cows would be required. According to the most aggressive time lines, ~41 months after microinjection, a single female transgenic animal would have produced ~20 kg of recombinant protein at the end of her first lactation period. For production of 100 kg, F1 offspring is required, which can be generated via in vitro fertilization of oocytes collected via nonsurgical ovum pick-up (OPU). The latter procedure can be completed before the heifer calf has reached 6 months of age. The transgenic female embryos can be selected based on the PCR analysis of a small biopsy, as described in a previous section. Thus, ~100 kg are obtained from transgenic F1 offspring ~56 months after successful microinjection of the transgene DNA. If a male transgenic animal is born, no milk will be available until female transgenic offspring is generated. Since sperm is not be available until the transgenic bull has reached sexual maturity, ~100 kg is obtained ~65 months after microinjection. In contrast, repeated nuclear transfer with expanded transgenic cells can instantly generate production herds, which consist exclusively of genetically identical F0 animals. Consequently, ~100 kg of recombinant protein is obtained ~41 months after nuclear transfer, exceeding any scenario based on microinjection. Since the induction of lactation of 5 calves at 6 months of age can yield well over 100 L of milk containing a total of >200 g of recombinant protein, product characterization and (pre)clinical development can be started even sooner, 15 months after nuclear transfer.

Given the advantages of preselection of transgenic embryos, genetic consistency, and reduced time of product development, it is not surprising that nuclear transfer is rapidly becoming the industry's technology of choice to generate transgenic animals for the production of biopharmaceuticals in milk. In fact, the combination of large protein quantities, safety, lower costs, and the reduced time of development, has made the transgenic animal platform even more attractive for production of therapeutic proteins.

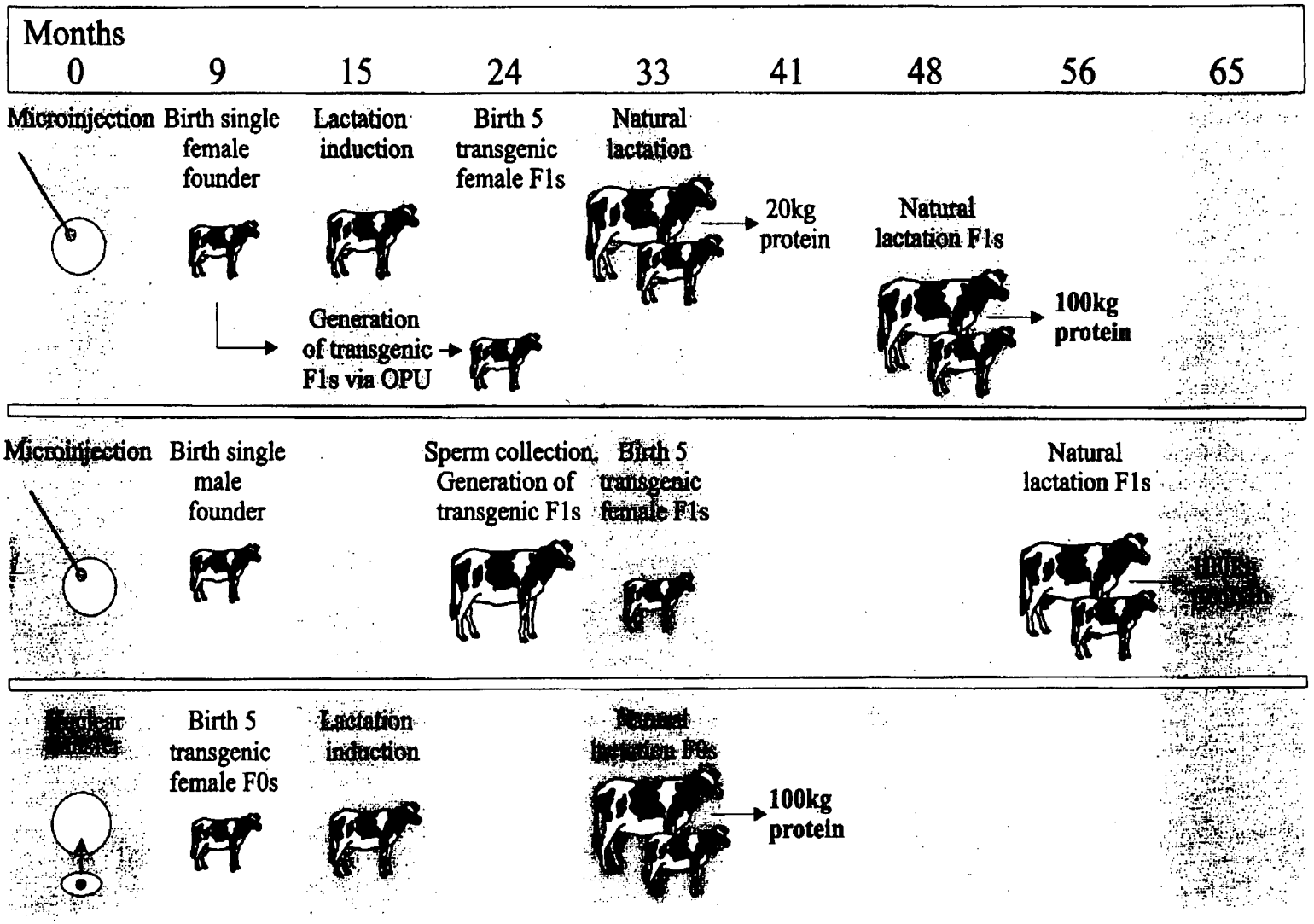

Figure 1. Three scenarios for the generation of transgenic cattle producing recombinant protein in milk. Time lines (in months) for the production of 100 kg of recombinant protein are outlined when the production herd is generated via microinjection and conventional breeding, or nuclear transfer. Calculations are based on the assumption that a single cow produces 10,000 L of milk over a lactation period of ~ 8 months. Concentration of recombinant protein in milk is 2 g/L.

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